Eur päisches Patentamt | European Patent Office | Office européen des brevets

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

95870124.5

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

D. BIEBER

[redacted]

21/11/96

Eur päisches Patentamt | Eur pean Patent Office | Office eur péen des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.:
Demande n°: 95870124.5

Anmeldetag:
Date of filing:
Date de dépôt: 21/11/95

Anmelder:
Applicant(s):
Demandeur(s):
EUROSCREEN S.A.
1160 Bruxelles
BELGIUM

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Receptor and nucleic acid molecule encoding said receptor

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C07K14/00

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/DE/DK/ES/[redacted]/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR.

Object of the present invention.

The present invention concerns a new receptor having a preference for pyrimidine nucleotides over purine nucleotides and the nucleic acid molecule encoding said receptor, vectors comprising said nucleic acid molecule, cells transformed by said vector, antibodies directed against said receptor, nucleic acid probes directed against said nucleic acid molecule, pharmaceutical compositions comprising said products and non human transgenic animals expressing the receptor according to the invention or the nucleic acid molecule according to said receptor.

The invention further provides methods for determining ligand binding, detecting expression, screening for drugs, molecular binding specifically to said receptor and treatment involving the receptor according to the invention.

Background of the invention.

The cloning of several receptors for ATP has been reported since 1993. In keeping with the latest nomenclature proposal, these P2 purinergic receptors can be subdivided into two classes: G protein-coupled receptors, or P2Y

receptors, and receptors with intrinsic ion channel activity or P2X receptors (2). Two distinct rat P2X receptors have been cloned, respectively from the vas deferens (3) and phaechromocytoma PC12 cells (4): they have a characteristic topology, with two hydrophobic putatively membrane-spanning segments and an ion pore motif reminiscent of potassium channels. In the P2Y family, the sequences of two subtypes, both coupled to phospholipase C, have been published: chick (5), turkey (6), bovine (7), mouse and rat (8) P2Y1 receptors (formerly called P2Y); murine (9,10), rat (11) and human (12) P2Y2 receptors (previously named P2U) on the other hand. In addition, a P2Y3 receptor, with a preference for ADP over ATP, has been cloned from chick brain, but its sequence is not yet published (13). Furthermore, the 6H1 orphan receptor, cloned from activated chicken T lymphocytes, exhibits a significant degree of homology to the P2Y1 and P2Y2 receptors, suggesting that it also belongs to the P2Y family, although its responsiveness to nucleotides has not yet been demonstrated (14).

Summary of the invention.

This invention provides a receptor having a preference for pyrimidine nucleotides over purine nucleotides. A receptor having a preference for pyrimidine nucleotides over purine nucleotides means a receptor for which pyrimidine nucleotides and purine nucleotides are not equally active and equipotent. This means that the receptor according to the invention in presence of these agonists presents a functional response (preferably the accumulation of Inositol triphosphate (IP3), diacyglycerol (DAG), or calcium ions) to lower concentration of pyrimidine nucleotides than to purine nucleotides or a more important functional response to similar concentration of pyrimidine

nucleotide than to purine nucleotide.

The inositol phosphate (IP3) accumulation after addition of said agonists is described in the specification thereafter.

Advantageously, the receptor according to the invention has at least twofold, preferably tenfold preference for pyrimidine nucleotides over purine nucleotides.

The receptor according to the invention is also characterized by a preference for uridine nucleotides over adenine nucleotides (preferably a preference for UTP and UDP over ATP and ADP).

The receptor according to the invention is also characterized by an equal preference (equipotent) for UTP and UDP.

The receptor according to the invention is a receptor, preferably a G protein-coupled receptor, which belongs structurally to the purinergic receptor family (P2Y family) but functionally is a pyrimidinergic receptor.

According to a preferred embodiment of the present invention, the receptor is a human receptor.

Said receptor has an amino acid sequence having more than 60% homology with the amino acid sequence shown in figure 1. Preferably, the amino acid sequence of the receptor according to the invention has at least the amino acid sequence shown in figure 1 or a portion thereof.

A portion of the amino acid sequence means a peptide or a protein having the same binding properties as the receptor according to the invention (i.e. peptide or a protein which is characterized by a preference for pyrimidine nucleotides over purine nucleotides).

The present invention is also related to a nucleic acid molecule, such as a DNA molecule or an RNA molecule,

encoding the receptor according to the invention.

Preferably, said DNA molecule is a cDNA molecule or a genomic DNA molecule.

Preferably, said nucleic acid molecule has more than 60% homology to the DNA sequence shown in figure 1.

Preferably, the nucleic acid molecule according to the invention is at least the DNA sequence shown in figure 1 or portion thereof. "A portion of a nucleic acid sequence" means a nucleic acid sequence encoding at least a portion of amino acid sequence as described above.

The present invention is also related to a vector comprising the nucleic acid molecule according to the invention. Preferably, said vector is adapted for expression in a cell and comprises the regulatory elements necessary for expressing the amino acid molecule in said cell operatively linked to the nucleic acid sequence according to the invention as to permit expression thereof.

Preferably, said cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells. The vector according to the invention is a plasmid or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

The plasmid may be the pcDNA3-P2Y4.

The present invention concerns also the cell (preferably a mammalian cell, such as a 1321N1 cell) transformed by the vector according to the invention. Advantageously, said cell is preferably non neuronal in origin and is chosen among the group consisting of a COS-7 cell, an LM(tk-) cell, an NIH-3T3 cell or a 1321N1 cell.

The present invention is also related to a nucleic acid probe comprising the nucleic acid molecule according to the invention, of at least 15 nucleotides capable of

specifically hybridizing with a unique sequence included in the sequence of the nucleic acid molecule encoding the receptor according to the invention. Said nucleic acid probe may be a DNA or an RNA molecule.

The invention concerns also an antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA molecule encoding the receptor according to the invention so as to prevent translation of said mRNA molecule or an antisense oligonucleotide having a sequence capable of specifically hybridizing to the cDNA molecule encoding the receptor according to the invention.

Said antisense oligonucleotide may comprise chemical analogs of nucleotide or substances which inactivate mRNA, or be included in an RNA molecule endowed with ribozyne activity.

Another aspect of the present invention concerns a ligand other than purine and pyrimidine nucleotides (preferably an antibody) capable of binding to a receptor according to the invention and an anti-ligand (preferably also an antibody) capable of competitively inhibiting the binding of said ligand to the receptor according to the invention.

Preferably, said antibody is a monoclonal antibody.

The present invention concerns also the monoclonal antibody directed to an epitope of the receptor according to the invention and present on the surface of a cell expressing said receptor.

The invention concerns also the pharmaceutical composition comprising an effective amount of oligonucleotide according to the invention, effective to decrease the activity of said receptor by passing through a cell membrane and binding specifically with mRNA encoding the receptor

according to the invention in the cell so as to prevent its translation. The pharmaceutical composition comprises also a pharmaceutically acceptable carrier capable of passing through said cell membrane.

Preferably, in said pharmaceutical composition, the oligonucleotide is coupled to a substance, such as a ribozyme, which inactivates mRNA.

Preferably, the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure. The structure of the pharmaceutically acceptable carrier in said pharmaceutical composition is capable of binding to a receptor which is specific for a selected cell type.

Preferably, said pharmaceutical composition comprises an amount of the antibody according to the invention effective to block the binding of a ligand to the receptor according to the invention and a pharmaceutically acceptable carrier.

The present invention concerns also a transgenic non human mammal overexpressing (or expressing ectopically) the nucleic acid molecule encoding the receptor according to the invention.

The present invention also concerns a transgenic non human mammal comprising a homologous recombination knockout of the native receptor according to the invention.

According to a preferred embodiment of the invention, the transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid according to the invention is so placed as to be transcripted into antisense mRNA which is complementary to the mRNA encoding the receptor according to the invention and

which hybridizes to mRNA encoding said receptor, thereby reducing its translation. Preferably, the transgenic non human mammal according to the invention comprises a nucleic acid molecule encoding the receptor according to the invention and comprises additionally an inducible promoter or a tissue specific regulatory element.

Preferably, the transgenic non human mammal is a mouse.

The invention relates to a method for determining whether a ligand can be specifically bound to the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to said receptor.

The invention relates to a method for determining whether a ligand can specifically bind to a receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said receptor. Preferably, said method is used when the ligand is not previously known.

The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises contacting a cell transfected

with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

The invention relates to a method for determining whether a ligand is an agonist of the receptor according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in second messenger concentration or a modification in the cellular metabolism, (preferably determined by the acidification rate of the culture medium) a decrease in the

receptor activity, thereby determining whether the ligand is a receptor antagonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the receptor according to the invention, which comprises preparing a cell extract from cells transfected with an expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a mofidication in the production of a second messenger, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

Preferably, the second messenger assay comprises measurement of intracellular cAMP, intracellular inositol phosphate (IP3), intracellular diacylglycerol (DAG) concentration or intracellular calcium mobilization.

Preferably, the cell used in said method is a mammalian cell non neuronal in origin, such as a COS-7 cell, a CHO cell, a LM(tk-) cell an NIH-3T3 cell or 1321N1.

In said method, the ligand is not previously known.

The invention is also related to the ligand isolated and detected by any of the preceding methods.

The present invention concerns also the pharmaceutical composition which comprises an effective amount of an agonist or an antagonist of the receptor according to the invention, effective to reduce the activity of said receptor and a pharmaceutically acceptable carrier.

For instance, said agonist or antagonist may be used in a pharmaceutical composition in the treatment of cystic fibrosis, and the method according to the invention

may be advantageously used in the detection of improved drugs which are used in the treatment of cystic fibrosis.

Therefore, the previously described methods may be used for the screening of drugs to identify drugs which specifically bind to the receptor according to the invention.

The invention is also related to the drugs isolated and detected by any of these methods.

The present invention concerns also a pharmaceutical composition comprising said drugs and a pharmaceutically acceptable carrier.

The invention is also related to a method of detecting expression of a receptor according to the invention by detecting the presence of mRNA coding for a receptor, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to the invention under hybridizing conditions and detecting the presence of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

Said hybridization conditions are stringent conditions.

The present invention concerns also the use of the pharmaceutical composition according to the invention for the treatment and/or prevention of cystic fibrosis.

The present invention concerns also a method for diagnosing a predisposition to a disorder associated with the activity of the receptor according to the invention. Said method comprises:

a) obtaining nucleic acid molecules of subjects suffering from said disorder;

b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;

c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;

d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;

e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to subjects suffering from said disorder;

f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and

g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

A last aspect of the present invention concerns a method of preparing the receptor according to the invention, which comprises:

a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;

b) inserting the vector of step a in a suitable host cell;

c) incubating the cell of step b under conditions allowing the expression of the receptor according to the invention;

d) recovering the receptor so obtained; and

e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

Short description of the drawings.

Figure 1 represents nucleotide and deduced amino acid sequence of the human $P2Y_4$ receptor according to the invention. The putative membrane-spanning domains are underlined and numbered I to VII. The consensus sequence conserved between all the P2Y receptors and the three amino acids (AHN) corresponding to the RGD sequence in the first extracellular loop of the $P2Y_2$ receptor are represented in bold. The putative phosphorylation sites by PKC or by calmodulin-dependent protein kinases and PKC are indicated respectively by black squares (■) and by open circles (O).

Figure 2 is a dendrogram representing structural relatedness among the cloned P2Y receptor and the closest neighbour in the G protein-coupled receptor family. The plot was constructed using the multiple sequence alignment program Pileup of the GCG package . For each sequence, the analysis takes into account a segment covering the first five putative membrane-spanning domains.

Figure 3 represents a northern blot analysis of $P2Y_4$ receptor expression. The Northern blot was performed with 15 μg of total RNA from human placenta and 4 μg of poly(A)+ RNA from K562 cells and from two different human placentas. The probe was the human $P2Y_4$ gene fragment amplified by PCR (TM2 to TM7).

Figure 4 represents concentration action curves comparing

the effect of adenine and uridine nucleotides on IP3 accumulation in $P2Y_4$ receptor-transfected 1321N1 cells. 1321N1 cells transfected with the $P2Y_4$ construct were assayed for the accumulation of IP_3 in response to various concentrations of the following nucleotides: UDP, UTP, ATP and ADP. The data represent the mean ± S.D. of triplicate experimental points and are representative of two independent experiments.

Detailed description of the invention.

EXPERIMENTAL PROCEDURES

1. Materials

Trypsin was from Flow Laboratories (Bioggio, Switzerland) and the culture media, reagents, G418, fetal calf serum (FCS), restriction enzymes and Taq polymerase were purchased from GIBCO BRL (Grand Island, NY). The radioactive products myo-D-[2-^{3}H]inositol (17.7 Ci/mmol) and [a^{32}P]ATP (800 Ci/mmol) were from Amersham (Ghent, Belgium). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Richmond, Calif.). UTP, UDP, ATP, ADP, carbachol, LiCl and apyrase grade VII were obtained from Sigma Chemical Co. (St. Louis, MO). 2MeSATP was from Research Biochemicals Inc. (Natick, MA). pcDNA3 is an expression vector developed by Invitrogen (San Diego, CA).

2. Cloning and sequencing

Degenerate oligonucleotide primers were synthesized on the basis of the best conserved segments between the murine P2Y2 and the chick P2Y1 receptor sequences. These primers were used to amplify novel receptor gene fragments by low-stringency PCR starting from human genomic DNA. The amplification conditions were as follows: 93 °C 1 min, 50 °C 2 min, 72 °C 3 min; 35 cycles. The PCR products with sizes

compatible with P2 receptor gene fragments were subcloned in M13mp18 and M13mp19 and sequenced by the Sanger dideoxy nucleotide chain termination method. One of the resulting clones sharing similarities with P2 receptors, was labelled by random priming and used to screen a human genomic DNA library constructed in the λ Charon 4a vector. The hybridization was in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M Sodium citrate) and 40% formamide at 42 °C for 14 h and the final wash conditions were 0.1 x SSC, 0.1% SDS at 65 °C A preparation of λ phages (15) was made for several clones which hybridized strongly with the probe. A restriction map and a Southern blotting analysis allowed to isolate a 1.4 kb NheI-EcoRV fragment that was subcloned into the pBluescript SK^+ vector (Stratagene). The complete sequence of the new receptor coding sequence was obtained on both strands after subcloning of overlapping fragments in M13mp18 and M13mp19.

3. Cell culture and transfection

The $P2Y_4$ receptor coding sequence was subcloned between the HindIII and the EcoRV sites of the pcDNA3 expression vector for transfection into 1321N1 human astrocytoma cells, a cell line which does not respond to nucleotides and which has already been used for the expression of purinergic receptors (6,12). Cells were transfected with the recombinant pcDNA3 plasmid (pcDNA3-$P2Y_4$) using the calcium phosphate precipitation method as described (16). 1321N1 cells were incubated for 6 hours at 37 °C in the presence of pcDNA3 vector alone or vector containing the $P2Y_4$ receptor coding sequence, then washed and incubated in culture medium (10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B in Dulbecco's modified Eagle's medium (DMEM)). The selection with G418 (400

μg/ml) was started two days after transfection. From the pool of transfected 1321N1 cells, individual clones were isolated by limiting dilution with the aim of selecting clones with high IP stimulation factors in response to nucleotides. The different clones were maintained in a medium containing 400 μg/ml G418.

4. Inositol phosphates (IP) measurement

1321N1 cells were labelled for 24 hours with 10 μCi/ml [^{3}H] inositol in inositol free DMEM containing 5% FCS, antibiotics, amphotericine and 2U/ml apyrase as described (6). Cells were washed twice with Krebs-Ringer Hepes (KRH) buffer of the following composition (124 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.45 mM CaCl2, 1.25 mM KH2PO4, 25 mM Hepes (pH:7.4) and 8 mM glucose) and incubated in the same medium supplemented with 10 mM LiCl for 5 min before the addition of the agonists. The incubation was stopped after 20 min by the addition of an ice cold 3% perchloric acid solution. IP were extracted and separated on Dowex columns as previously described (17).

5. Northern blot and Southern blot analysis

Total and poly(A)$^+$ RNA were prepared from different tissues and human cell lines using the guanidinium thiocyanate-cesium chloride procedure (15), denatured by glyoxal and fractionated by electrophoresis on a 1% agarose gel in 10 mM phosphate buffer pH 7.0. DNA samples, prepared from the λ Charon 4a clones, were digested with restriction enzymes. Northern and Southern blots were prepared (15) and baked for 90 min at 80 °C. Membranes were prehybridized for at least 4 hours and hybridized overnight with the same probe as for the screening, at 42 °C in a solution containing 50% formamide for Northern blots and 40% formamide for Southern blots. Filters were washed twice for 15 min in 2 x SSC at

room temperature and then twice for 30 min in 0.2 x SSC at 60 °C before being exposed at -70 °C in the presence of intensifying screens for 5 days (Northern blots) or 1 hour (Southern blots).

RESULTS

1. Cloning and sequencing

In order to isolate new subtypes of P2 receptors, sets of degenerate oligonucleotide primers were synthesized on the basis of the best conserved segments in the published sequences of the chick brain P2Y1 (5) and murine neuroblastoma P2Y2 (9) receptors. These primers were used in low-stringency PCR on human genomic DNA as described (18). Some combinations generated discrete bands with a size compatible with that expected for P2 receptors. For example, the primer [5'-CAGATCTAGATA(CT)ATGTT(CT)(AC)A(CT)(CT)T(ACGT)GC-3'] corresponding to the second transmembrane region and the primer [5'-TCTTAAGCTTGG(AG)TC(ACGT)A(CG)(AG)CA(AG)CT(AG)TT-3'] corresponding to the seventh transmembrane region amplified a 712 bp fragment. The partial sequences obtained after sequencing were translated into peptidic sequences and compared to a local databank which contains G protein-coupled receptor sequences. Most of the clones resulting from these PCR products encoded a part of a new receptor which displayed 58% identity with the murine P2Y2 receptor and 42% identity with the chick P2Y1 receptor partial sequences. In addition, some clones encoded a peptidic sequence presenting 87% identity with the chick P2Y1 receptor and are therefore believed to represent fragments of the human P2Y1 gene.

The partial sequence of the new receptor was used as a probe to screen a human genomic DNA library. Several

clones that strongly hybridized with the probe at high stringency conditions were obtained and purified. The inserts of the clones varied from 12 to 17 kb and restriction analysis revealed that all clones belonged to a single locus. The full sequence of a 1.4 kb NheI-EcoRV fragment was obtained and an intronless open reading frame of 1095 bp was identified. The sequence is depicted in figure 1 where the putative membrane-spanning domains are underlined and numbered I to VII. The predicted molecular weight of the encoded protein is 36.5 kDa. This molecular weight is unlikely to be modified in vivo, since no N-glycosylation consensus sequences are found in the putative exofacial regions. In contrast with the human P2Y2 receptor, there is no RGD motif, an integrin binding consensus sequence, in the putative first extracellular loop. The three amino acid (AHN) corresponding to the RGD sequence in the first extracellular loop of the P2Y2 receptor are represented in bold in figure 1. Some potential sites of phosphorylation by protein kinase C (PKC) or by calmodulin-dependent protein kinases were identified in the third intracellular loop and in the carboxyterminal part of the receptor. The putative phosphorylation sites by PKC or by calmodulin-dependent protein kinases and PKC are indicated respectively by black squares and by open circles in figure 1. The four positively charged amino acid which have been reported to play a role in the P2Y2 receptor activation by ATP and UTP (1) are conserved in the P2Y4 sequence: His^{262}, Arg^{265}, Lys^{289} and Arg^{292} (Figure 1). The P2Y4 amino acid sequence was compared to the chick P2Y1 and the murine P2Y2 amino acid sequences and to their closest neighbours in the G protein-coupled receptor family (Figure 2). The plot was constructed using the multiple sequence alignment program Pileup of the GCG package

(26). For each sequence, the analysis takes into account a segment covering the first five putative membrane-spanning domains. It is clear that, from a structural point of view, the newly cloned receptor is more closely related to the human P2Y2 receptor (51% of identity between the complete sequences) than to the chick P2Y1 receptor (35%).

2. Tissue distribution of the P2Y4 receptor

The tissue distribution of P2Y4 transcripts was investigated by Northern blotting. A number of rat tissues (heart, brain, liver, testis and kidney) were tested using a human probe at low stringency, but no hybridization signal could be obtained. No P2Y4 transcript could be detected in the following human cell lines: K562 leukemia cells (Figure 3), HL-60 leukemia cells and SH-SY5Y human neuroblastoma cells. The Northern blot was performed with 15 μg of total RNA from human placenta and 4 μg of poly(A)$^+$ RNA from K562 cells and from two different human placentas. The probe was the human P2Y4 gene fragment amplified by PCR (TM2 to TM7). On the contrary, a strong signal, corresponding to a 1.8 kb mRNA, was found in human placenta (Figure 3).

3. Functional expression of the new P2 receptor in 1321N1 cells

After transfection of the pcDNA3-P2Y4 construction in 1321N1 cells, the pool of G418-resistant clones was tested for their functional response (IP3 accumulation) to ATP and UTP. Both nucleotides were found to be agonists of the P2Y4 receptor, but the response to UTP was more robust. About 20 transfected clones were then isolated and tested for their response to UTP. The clone presenting the highest IP accumulation factor in response to UTP was selected and used in all subsequent experiments. Figure 4 represents concentration-action curves comparing the effect of adenine

and uridine nucleotides on IP_3 accumulation in P2Y4 receptor-transfected 1321N1 cells. 1321N1 cells transfected with the P2Y4 construct were assayed for the accumulation of IP_3 in response to various concentration of UDP, UTP, ATP and ADP. The data represent the mean ± of S.D. of triplicate experimental points and are representative of two independent experiments. UTP and UDP were found to be the most important agonists, producing the same maximal effect with similar potency (EC_{50} around 2μM). ATP had a lower potency (EC_{50} around 20μM) and its maximal effect was only 35% of that of UTP or UDP (mean of four independent experiments). The effect of ADP was barely detectable and 2-methyl thio ATP was completely inactive.

The Inventor describes herein the cloning of a human gene encoding a novel member of the P2 purinergic receptor family.

The deduced amino acid sequence is consistent with a G protein-coupled receptor and exhibits 51% identity with the human P2Y2 receptor and 35% with the chick P2Y1 receptor. This receptor is provisionally named P2Y4. No N-glycosylation consensus sequence can be detected in the putative exofacial regions. This situation is rare in G protein-coupled receptors but not unique: it has also been reported for the rat α2B-adrenergic receptor (19), a human receptor for the LD78 chemokine (20) and the rat RBS11 orphan receptor (21). Despite its structural relatedness to the human P2Y2 receptor, the newly cloned receptor exhibits one conspicuous difference in the putative first extracellular loop: the RGD motif, an integrin binding consensus sequence, present in the P2Y2 receptor is not conserved, although clusters of four amino acids are perfectly conserved on both sides. Site-directed mutagenesis studies have demonstrated

the crucial influence of charged amino acid in transmembrane helices 6 and 7 of the P2Y2 receptor on agonist potency and specificity (1). His^{262}, Arg^{265} and Arg^{292} seem to be directly involved in the binding of the negatively charged phosphate groups, whereas a mutation of Lys^{289} into Arg decreases the affinity for ATP/UTP and increases that for ADP/UDP. These four residues are conserved in the P2Y4 subtype, whereas Arg^{265} and Lys^{289} are replaced respectively by Lys and Gln in the P2Y1 receptor (5). The P2Y4 receptor sequence presents also a series of 10 amino acid (LFLTCISVHR) which is a consensus sequence between all the P2Y receptors cloned until now. Potential sites of phosphorylation by protein kinase C (PKC) or by calmodulin-dependent protein kinases were identified in the third intracellular loop and in the carboxyterminal part of the receptor. These regions are also particularly rich in serine and threonine residues that could be targets for the family of G protein coupled receptor kinases. Northern Blot analysis detected a strong signal corresponding to a 1.8 kb mRNA in human placenta. Various rat tissues tested at low stringency and several human cell lines were negative. This suggests a rather restricted expression of the P2Y4 receptor.

In the late eighties, it became apparent that extracellular uridine nucleotides exert effects on many tissues and cells. It was then proposed that these actions are mediated by pyrimidinoceptors distinct from the purinoceptors involved in the response to adenine nucleotides (22). The existence of nucleotide receptors common to ATP and UTP constituted an alternative possibility, in favor of which experimental evidence started to accumulate, for instance the lack of additivity and cross-desensitization of the responses to the two nucleotides (23,24). The final demonstration of

this concept was provided by the cloning and functional expression of the P2U/P2Y2 receptor at which ATP and UTP are equally active and equipotent (9,10,12). However the pyrimidinoceptor hypothesis reemerged with the observation that UTP and UDP stimulate inositol phosphates' formation in C6-2B rat glioma cells, whereas ATP and ADP are totally inactive (25). However the C6 glioma cells, from which the C6-2B subclone was isolated, express a typical P2U response: a mutation of that receptor, with selective loss of the adenine nucleotides responsiveness, might thus explain the peculiar behavior of C6-2B cells. The present results conclusively demonstrate the existence of a human gene encoding a receptor with a preference for pyrimidine over purine nucleotides. Furthermore they show on the basis of its structure that this receptor belongs to the P2Y family, which thus encompass selective purinoceptors (P2Y1), nucleotide receptors responsive to both adenine and uridine nucleotides (P2Y2) and pyrimidinoceptors (P2Y4).

REFERENCES

1. Erb, L., Garrad, R., Wang, Y., Quinn, T., Turner, J.T., and Weisman, G.A. (1995) J. Biol. Chem. **270**, 4185-4188.

2. Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., and Williams, M. (1994) Pharm. Rev. **46**, 143-156.

3. Valera, S., Hussy, N., Evans, R.J., Adami, N., North, R.A., Surprenant, A., and Buell, G. (1994) Nature **371**, 516-519.

4. Brake, A.J., Wagenbach, M.J., and Julius, D. (1994) Nature **371**, 519-523.

5. Webb, T.E., Simon, J., Krishek, B.J., Bateson, A.N., Smart, T.G., King, B.F., Burnstock, G., and Barnard, E.A. (1993) FEBS **324**, 219-225.

6. Filtz, T.M., Li, Q., Boyer, J.L., Nicholas, R.A., and Harden, T.K. (1994) Mol. Pharm. **46**, 8-14.

7. Henderson, D.J., Elliot, D.G., Smith, G.M., Webb, T.E., and Dainty, I.A. (1995) Biochem. Biophys. Res. Commun. **212**, 648-656.

8. Tokoyama, Y., Hara, M., Jones, E.M.C., Fan, Z., and Bell, G.I. (1995) Biochem. Biophys. Res. Commun. **211**, 211-218.

9. Lustig, K.D., Shiau, A.K., Brake, A.J., and Julius, D.

(1993) Proc. Natl. Acad. Sci. **90**, 5113-5117.

10. Erb, L., Lustig, K.D., Sullivan, D.M., Turner, J.T., and Weisman, G.A. (1993) Proc Natl Acad Sci **90**, 10449-10453.

11. Rice, W.R., Burton, F.M., and Fiedeldey, D.T. (1995) Am. J. Respir. Cell, Molec. Biol. **12**, 27-32.

12. Parr, C.E., Sullivan, D.M., Paradiso, A.M., Lazarowski, E.R., Burch, L.H., Olsen, J.C., Erb, L., Weisman, G.A., Boucher, R.C., and Turner, J.T. (1994) Proc. Natl. Acad. Sci. **91**, 3275-3279.

13. Barnard, E.A., Burnstock, G., and Webb, T.E. (1994) TiPS **15**, 67-70.

14. Kaplan, M.H., Smith, D.I., and Sundick, R.S. (1993) J. Immun. **151**, 628-636.

15. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY).

16. Velu, T.J., Beguinot, L., Vass, W.C., Zhang, K., Pastan, I., and Lowry, D.R. (1989) J. Cell. Biochem. **39**, 153-166.

17. Communi, D., Raspe, E., Pirotton, S., and Boeynaems, J.M. (1995) Circ. Res. **76**, 191-198.

18. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M.J., Dumont, J.E., and Vassart, G. (1989) Science **244**, 569-572.

19. Zeng, D., Harrison, J.K., D'Angelo, D.D., Barber, C.M., Tucker, A.L., Lu, Z., and Lynch, K.R. (1990) Proc. Natl. Acad. Sci. **87**, 3102-3106.

20. Nomura, H., Nielsen, B.W., and Matsushima, K. (1993) Int. Immun. **5**, 1239-1249.

21. Harrison, J.K., Barber, C.M., and Lynch, K.R. (1994) Neuroscience Letters **169**, 85-89.

22. Seifert, R. and Schultz, G. (1989) TiPS **10**, 365-369.

23. Brown, H.A., Lazarowski, E.R., Boucher, R.C., and Harden, T.K. (1991) Mol. Pharm. **40**, 648-655.

24. O'Connor, S.E., Dainty, I.A., and Leff, P. (1991) TiPS **12**, 137-141.

25. Lazarowski, E.R. and Harden, T.K. (1994) J. Biol. Chem. **269**, 11830-11836.

26. Devereux, J., Haeberli, P. and Smithies O.A. (1984) Nucleic Acids Res. **12**, 387-395.

CLAIMS.

1. Receptor having a preference for pyrimidine nucleotides over purine nucleotides.

2. Receptor according to claim 1, having at least a twofold preference, preferably tenfold preference for pyrimidine nucleotides over purine nucleotides.

3. Receptor according to claim 1 or 2, having a preference for uridine nucleotides over adenine nucleotides.

4. Receptor according to claim 3, having a preference for UTP and UDP over ATP and ADP.

5. Receptor according to any of the preceding claims, which belongs to the P2 receptor family.

6. Receptor according to claim 5, being a G protein-coupled receptor.

7. Receptor according to any of the preceding claims, being a human receptor.

8. Receptor according to any of the preceding claims, which has an amino acid sequence having more than 60% homology with the amino acid sequence shown in Figure 1.

9. Receptor according to any of the preceding claims, which has at least the amino acid sequence shown in Figure 1 or portion thereof.

10. Nucleic acid molecule encoding the receptor according to any of the preceding claims.

11. Nucleic acid molecule according to claim 10, wherein the nucleic acid molecule is DNA or RNA molecule.

12. DNA molecule according to claim 11, which is a cDNA molecule or a genomic DNA molecule.

13. Nucleic acid molecule according to any of the claims 10 to 12, having more than 60% homology to the DNA sequence shown in Figure 1.

14. DNA molecule according to claim 13, which has

at least the DNA sequence as shown in figure 1 or a portion thereof.

15. Vector comprising the nucleic acid molecule according to any of the claims 10 to 14.

16. Vector of claim 15, adapted for expression in a cell, which comprises the regulatory elements necessary for expression of the nucleic acid molecule in said cell operatively linked to the nucleic acid molecule according to any of the claims 10 to 14 as to permit expression thereof.

17. Vector of claim 16, wherein the cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells.

18. Vector according to any of the claims 15 to 17, wherein the vector is a plasmid or a virus, preferably a baculovirus, an adenovirus or a Semliki Forest virus.

19. Vector of claim 18, wherein the plasmid is pcDNA3-P2Y4.

20. Cell comprising the vector according to any of the claims 15 to 19.

21. Cell of claim 20, wherein the cell is a mammalian cell, preferably non neuronal in origin.

22. Cell of claim 22, wherein the cell is chosen among the group consisting of COS-7 cells, LM(tk-) cells, NIH-3T3 cells or 1321N1 cells.

23. Nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the nucleic acid molecule according to any of the claims 10 to 14.

24. Nucleic acid probe of claim 23, wherein the nucleic acid is DNA or RNA.

25. Antisense oligonucleotide having a sequence

capable of specifically hybridizing to a mRNA molecule of claim 11, so as to prevent translation of the mRNA molecule.

26. Antisense oligonucleotide having a sequence capable of specifically hybridizing to the DNA molecule of claim 12.

27. Antisense oligonucleotide according to claim 25 or 26, comprising chemical analogs of nucleotides.

28. Ligand other than purine and pyridine nucleotides capable of binding to a receptor according to any of the claims 1 to 9.

29. Anti-ligand capable of competitively inhibiting the binding of the ligand according to claim 28 to the receptor according to any of the claims 1 to 9.

30. Ligand according to claim 28 which is an antibody.

31. Anti-ligand according to claim 29 which is an antibody.

32. Antibody according to claim 30 or 31, which is a monoclonal antibody.

33. Monoclonal antibody according to claim 32, directed to an epitope of the receptor according to any of the claims 1 to 9, present on the surface of a cell expressing said receptor.

34. Pharmaceutical composition comprising an amount of the oligonucleotide according to claim 25, effective to decrease activity of the receptor according to any of the claims 1 to 9 by passing through a cell membrane and binding specifically with mRNA encoding said receptor in the cell so as to prevent its translation, and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

35. Pharmaceutical composition of claim 34,

wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

36. Pharmaceutical composition of claim 35, wherein the substance which inactivates mRNA is a ribozyme.

37. Pharmaceutical composition according to any of the claims 34 to 36, wherein the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure.

38. Pharmaceutical composition of claim 37, wherein the structure of the pharmaceutically acceptable carrier is capable of binding to a receptor which is specific for a selected cell type.

39. Pharmaceutical composition which comprises an effective amount of the anti-ligand of claim 29, effective to block binding of a ligand to the receptor according to any of the claims 1 to 9 and a pharmaceutically acceptable carrier.

40. Transgenic non human mammal expressing the nucleic acid molecule according to any of the claims 10 to 14.

41. Transgenic non human mammal comprising a homologous recombination knockout of the native receptor according to any of the claims 1 to 9.

42. Transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid molecule according to any of the claims 10 to 14 so placed as to be transcripted into antisense mRNA which is complementary to the mRNA of claim 11 and which hybridizes to said mRNA thereby reducing its translation.

43. Transgenic non human mammal according to any of the claims 40 to 42, wherein the nucleic acid according

to any of the claims 10 to 14 additionally comprises an inducible promoter.

44. Transgenic non human mammal according to any of the claims 40 to 43, wherein the nucleic acid according to claim 10 to 14 additionally comprises tissue specific regulatory elements.

45. Transgenic non human mammal according to any of the claims 40 to 44, which is a mouse.

46. Method for determining whether a ligand can specifically bind to a receptor according to any of the claims 1 to 9, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand under conditions permitting binding of ligand to such receptor and detecting the presence of any such ligand bound specifically to said receptor, thereby determining whether the ligand binds specifically to said receptor.

47. Method for determining whether a ligand can specifically bind to the receptor according to any of the claims 1 to 9, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such receptor and detecting the presence of any ligand bound to said receptor, thereby determining whether the compound is capable of specifically binding to said receptor.

48. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 9, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said

receptor with the ligand under conditions permitting the activation of a functional receptor response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

49. Method for determining whether a ligand is an agonist of the receptor according to any of the claims 1 to 9, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, an increase in the receptor activity, thereby determining whether the ligand is a receptor agonist.

50. Method for determining whether a ligand is an antagonist of the receptor according to any of the claims 1 to 9, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in a second messenger concentration or a modification in the cellular metabolism, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

51. Method for determining whether a ligand is an antagonist of the receptor according to any of the claims 1 to 9, which comprises preparing a cell extract from cells

transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response and detecting by means of a bio-assay, such as a modification in a second messenger concentration, a decrease in the receptor activity, thereby determining whether the ligand is a receptor antagonist.

52. A method according to any of the claims 48 to 51, wherein the second messenger assay comprises measurement of intra-cellular cAMP, intra-cellular Inositol phosphate, intra-cellular diacylglycerol concentration or intra-cellular calcium mobilization.

53. Method according to any of the preceding claims 46 to 52, wherein the cell is a mammalian cell, preferably non neuronal in origin, and chosen among the group consisting of COS-7 cells, CHO cells, LM(tk-) cells, NIH-3T3 cells or 1321N1 cells.

54. Method according to any of the preceding claims 46 to 53, wherein the ligand is not previously known.

55. Ligand detected by the method according to any of the preceding claims 46 to 54.

56. Pharmaceutical composition which comprises the ligand according to claim 55 and a pharmaceutically acceptable carrier.

57. Method of screening drugs to identify drugs which specifically bind to the receptor according to any of the claims 1 to 9 on the surface of the cell, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs under conditions permitting binding of said drugs

to the receptor, and determining those drugs which specifically bind to the transfected cell, thereby identifying drugs which specifically bind to the receptor.

58. Method of screening drugs to identify drugs which specifically bind to the receptor according to any of the claims 1 to 9 on the surface of the cell, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cells extract, contacting the membrane fraction with a plurality of drugs and determining those drugs which bind to the transfected cell, thereby identifying drugs which specifically bind to said receptor.

59. Method of screening drugs to identify drugs which act as agonists of the receptor according to any of the claims 1 to 9, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs under conditions permitting the activation of a functional receptor response, and determining those drugs which activate such receptor using a bio-assay, such as a modification in a second messenger concentration or modification in the cellular metabolism, thereby identifying drugs which act as receptor agonists.

60. Method of screening drugs to identify drugs which act as agonists of the receptor according to any of the claims 1 to 9, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs under conditions permitting the activation of a functional receptor response,

and determining those drugs which activate such receptor using a bio-assay, such as a modification in a second messenger concentration, thereby identifying drugs which act as receptor agonists.

61. Method of screening drugs to identify drugs which act as antagonists of the receptor according to any of the claims 1 to 9, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said receptor with a plurality of drugs in the presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response, and determining those drugs which inhibit the activation of the receptor using a bio-assay, such as a modification in a second messenger concentration or modification in the cellular metabolism, thereby identifying drugs which act as receptor antagonists.

62. Method of screening drugs to identify drugs which act as antagonists of the receptor according to any of the claims 1 to 9, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs in presence of a known receptor agonist, under conditions permitting the activation of a functional receptor response, and determining those drugs which inhibit the activation of the receptor using a bio-assay, such as a modification in a second messenger concentration, thereby identifying drugs which act as receptor antagonists.

63. Drug detected by any of the methods according to claims 57 to 63.

64. Pharmaceutical composition comprising a drug

according to claim 63.

65. Method of detecting the expression of the receptor according to any of the claims 1 to 9, by detecting the presence of mRNA coding said receptor, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to claim 25 under hybridizing conditions, and detecting the presence of mRNA hybridized to the probe, thereby detecting the expression of the receptor by the cell.

66. Method of detecting the presence of the receptor according to any of the claims 1 to 9 on the surface of a cell, which comprises contacting the cell with the antibody of claim 30 under conditions permitting binding of the antibody to the receptor, and detecting the presence of the antibody bound to the cell, thereby detecting the presence of the receptor on the surface of the cell.

67. Method of determining the physiological effects of expressing varying levels of the receptor according to any of the claims 1 to 9, which comprises producing a transgenic non human mammal according to any of the claims 40 to 45 whose levels of receptor expression are varied by use of an inducible promoter which regulates the receptor expression.

68. Method of determining the physiological effects of expressing varying levels of the receptor according to any of the claims 1 to 9, which comprises producing a panel of transgenic non human mammals according to any of the claims 40 to 45, each expressing a different amount of said receptor.

69. Method for identifying an antagonist of the receptor according to any of the claims 1 to 9 capable of alleviating an abnormality in a subject wherein the

abnormality is alleviated by decreasing the activity of the receptor, which comprises administering the antagonist to a transgenic non human mammal according to any of the claims 40 to 45 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal as a result of receptor activity, thereby identifying the antagonist.

70. Antagonist identified by the method of claim 69.

71. Pharmaceutical composition comprising an antagonist according to claim 70 and a pharmaceutically acceptable carrier.

72. Method for identifying an agonist of the receptor according to any of the claims 1 to 9 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by activation of said receptor, which comprises administering the agonist to a transgenic non human mammal according to any of the claims 40 to 45 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal, the alleviation of the abnormalities indicating the identification of the agonist.

73. Agonist identified by the method of claim 72.

74. Pharmaceutical composition comprising an agonist according to claim 73 and a pharmaceutically acceptable carrier.

75. Method for diagnosing a predisposition to a disorder associated with the activity of a specific allele of the receptor according to any of the claims 1 to 9, which comprises:

a) obtaining nucleic acid molecules of subjects suffering from said disorder;

b) performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;

c) electrophoretically separating the resulting nucleic acid fragments on a sized gel;

d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to said nucleic acid molecule and labelled with a detectable marker;

e) detecting labelled bands which have hybridized to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern specific to subjects suffering from said disorder;

f) preparing nucleic acid molecules obtained for diagnosis by step a-e; and

g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

76. Method of preparing the purified receptor according to any of the claims 1 to 9, which comprises:

a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said receptor so as to permit expression thereof, wherein the cell is selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells;

b) inserting the vector of step a in a suitable host cell;

c) incubating the cell of step b under conditions allowing the expression of the receptor according to the

invention;

d) recovering the receptor so obtained; and

e) purifying the receptor so recovered, thereby preparing an isolated receptor according to the invention.

6

ABSTRACT

RECEPTOR AND NUCLEIC ACID MOLECULE ENCODING SAID RECEPTOR.

The present invention concerns a new receptor having a preference for pyrimidine over purine nucleotides.

(Figure 4)

```
                AAGGGAGCTTGGGTAGGGGCCAGGCTAGCCTGAGTGCACCCAGATGCGCTTCTGTCAGCT   60
                CTCCCTAGTGCTTCAACCACTGCTCTCCCTGCTCTACTTTTTTTGCTCCAGCTCAGGGAT  120
                GGGGGGTGGGCAGGGAAATCCTGCCACCCTCACTTCTCCCCTTCCCATCTCCAGGGGGGCC  180

1               ATGGCCAGTACAGAGTCCTCCCTGTTGAGATCCCTAGGCCTCAGCCCAGGTCCTGGCAGC  240
1                M  A  S  T  E  S  S  L  L  R  S  L  G  L  S  P  G  P  G  S

61              AGTGAGGTGGAGCTGGACTGTTGGTTTGATGAGGATTTCAAGTTCATCCTGCTGCCTGTG  300
21               S  E  V  E  L  D  C  W  F  D  E  D  F  K  F  I  L  L  P  V

121             AGCTATGCAGTTGTCTTTGTGCTGGGCTTGGGCCTTAACGCCCCAACCCTATGGCTCTTC  360
41               S  Y  A  V  V  F  V  L  G  L  G  L  N  A  P  T  L  W  L  F
                                        I
181             ATCTTCCGCCTCCGACCCTGGGATGCAACGGCCACCTACATGTTCCACCTGGCATTGTCA  420
61               I  F  R  L  R  P  W  D  A  T  A  T  Y  M  F  H  L  A  L  S

241             GACACCTTGTATGTGCTGTCGCTGCCCACCCTCATCTACTATTATGCAGCCCACAACCAC  480
81               D  T  L  Y  V  L  S  L  P  T  L  I  Y  Y  Y  A  A  H  N  H
                                    II
301             TGGCCCTTTGGCACTGAGATCTGCAAGTTCGTCCGCTTTCTTTTCTATTGGAACCTCTAC  540
101              W  P  F  G  T  E  I  C  K  F  V  R  F  L  F  Y  W  N  L  Y

361             TGCAGTGTCCTTTTCCTCACCTGCATCAGCGTGCACCGCTACCTGGGCATCTGCCACCCA  600
121              C  S  V  L  F  L  T  C  I  S  V  H  R  Y  L  G  I  C  H  P
                    III
421             CTTCGGGCACTACGCTGGGGCCGCCCTCGCCTCGCAGGCCTTCTCTGCCTGGCAGTTTGG  660
141              L  R  A  L  R  W  G  R  P  R  L  A  G  L  L  C  L  A  V  W

481             TTGGTCGTAGCCGGCTGCCTCGTGCCCAACCTGTTCTTTGTCACAACCAGCAACAAAGGG  720
161              L  V  V  A  G  C  L  V  P  N  L  F  F  V  T  T  S  N  K  G
                    IV
541             ACCACCGTCCTGTGCCATGACACCACTCGGCCTGAAGAGTTTGACCACTATGTGCACTTC  780
181              T  T  V  L  C  H  D  T  T  R  P  E  E  F  D  H  Y  V  H  F

601             AGCTCGGCGGTCATGGGGCTGCTCTTTGGCGTGCCCTGCCTGGTCACTCTTGTTTGCTAT  840
201              S  S  A  V  M  G  L  L  F  G  V  P  C  L  V  T  L  V  C  Y
                                                V

661             GGACTCATGGCTCGTCGCCTGTATCAGCCCTTGCCAGGCTCTGCACAGTCGTCTTCTCGC  900
221              G  L  M  A  R  R  L  Y  Q  P  L  P  G  S  A  Q  S  S  S  R
                                                            ■

721             CTCCGCTCTCTCCGCACCATAGCTGTGGTGCTGACTGTCTTTGCTGTCTGCTTCGTGCCT  960
241              L  R  S  L  R  T  I  A  V  V  L  T  V  F  A  V  C  F  V  P
                    O                                           VI

781             TTCCACATCACCCGCACCATTTACTACCTGGCCAGGCTGTTGGAAGCTGACTGCCGAGTA 1020
261              F  H  I  T  R  T  I  Y  Y  L  A  R  L  L  E  A  D  C  R  V

841             CTGAACATTGTCAACGTGGTCTATAAAGTGACTCGGCCCCTGGCCAGTGCCAACAGCTGC 1080
281              L  N  I  V  N  V  V  Y  K  V  T  R  P  L  A  S  A  N  S  C
                                                            VII

901             CTGGATCCTGTGCTCTACTTGCTCACTGGGGACAAATATCGACGTCAGCTCCGTCAGCTC 1140
301              L  D  P  V  L  Y  L  L  T  G  D  K  Y  R  R  Q  L  R  Q  L

961             TGTGGTGGTGGCAAGCCCCAGCCCCGCACGGCTGCCTCTTCCCTGGCACTAGTGTCCCTG 1200
321              C  G  G  G  K  P  Q  P  R  T  A  A  S  S  L  A  L  V  S  L

1021            CCTGAGGATAGCAGCTGCAGGTGGGCGGCCACCCCCCAGGACAGTAGCTGCTCTACTCCT 1260
341              P  E  D  S  S  C  R  W  A  A  T  P  Q  D  S  S  C  S  T  P
                          ■                                             ■

1081            AGGGCAGATAGATTGTAACACGGGAAGCCGGGAAGTGAGAGAAAAGGGGATGAGTGCAGG 1320
361              R  A  D  R  L  *

                GCAGAGGTGAGGGAACCCAATAGTGATACCTGGTAAGGTGCTTCTTCCTCTTTTCCAGGC 1380
                TCTGGAGAGAAGCCCTCACCCTGAGGGTTGCCAGGGAGGCAGGGATATC            1429
```

FIG.1

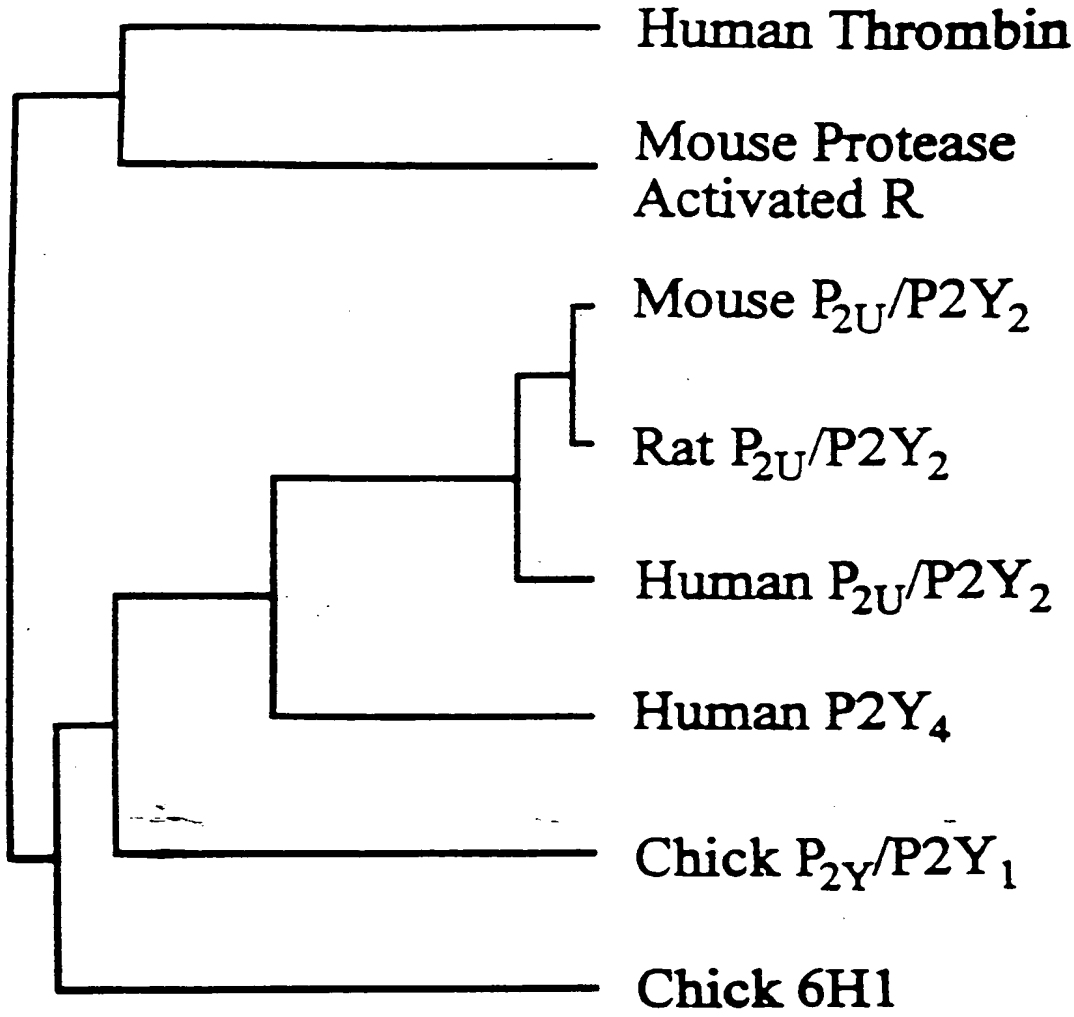
Human Thrombin
Mouse Protease Activated R
Mouse P_{2U}/P2Y_2
Rat P_{2U}/P2Y_2
Human P_{2U}/P2Y_2
Human P2Y_4
Chick P_{2Y}/P2Y_1
Chick 6H1

FIG.2

Expression of $P2Y_4$ receptors

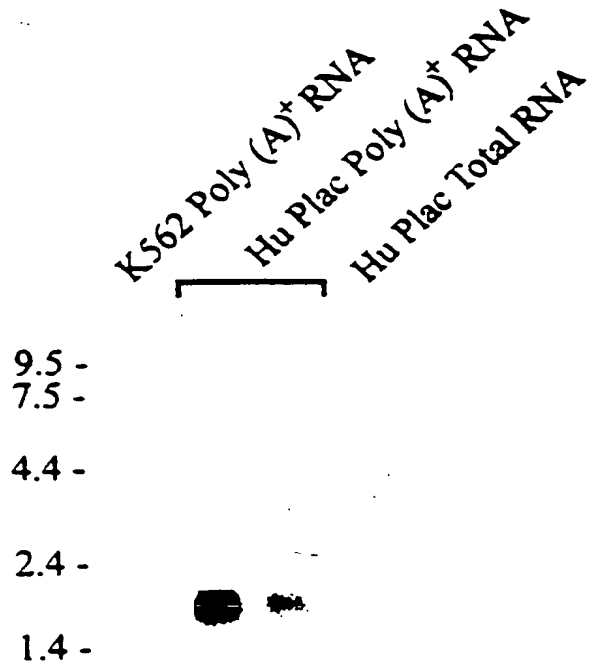

FIG.3

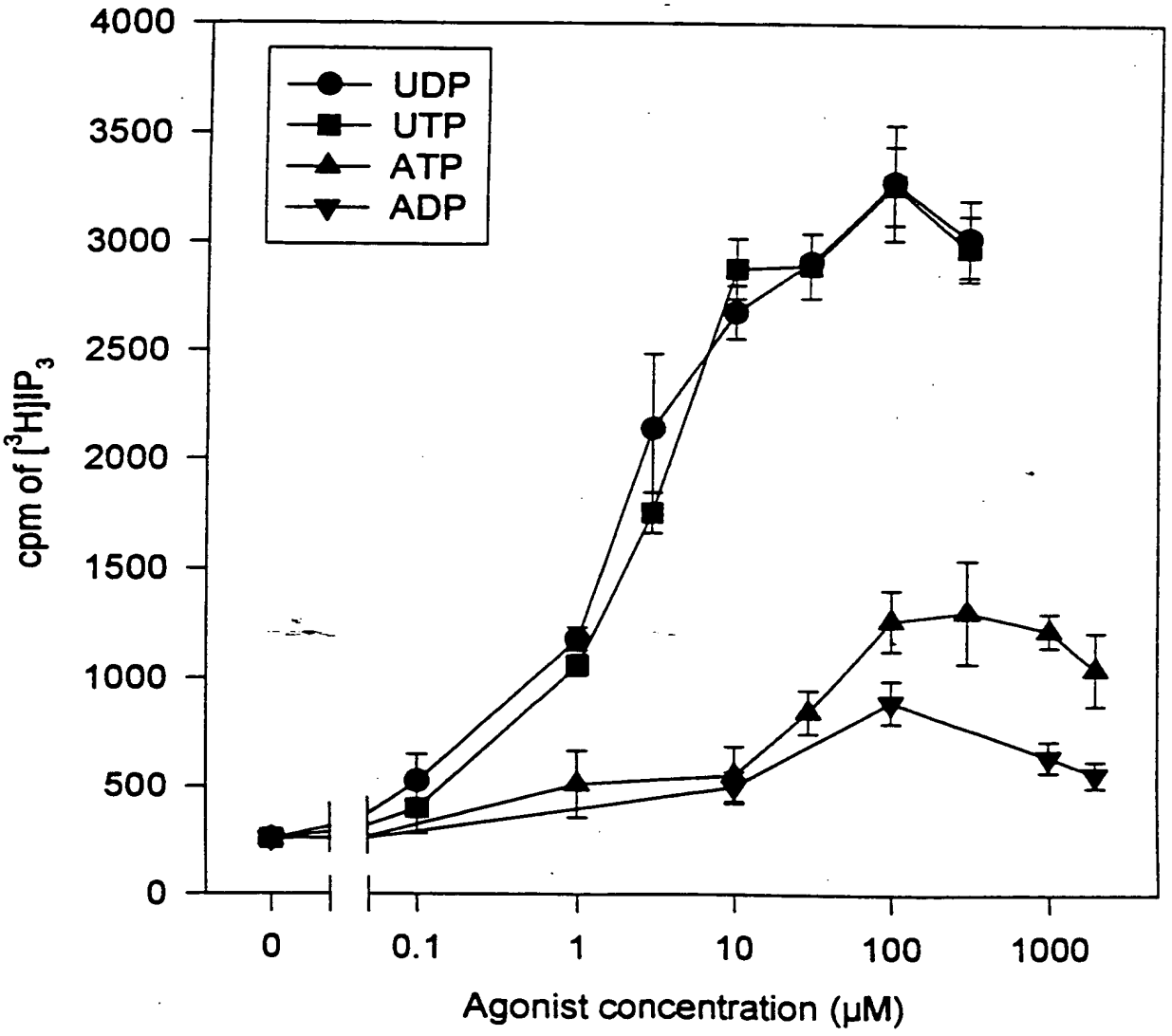
4000
3500
3000
2500
2000
1500
1000
500
0
cpm of [^{3}H]IP$_3$
UDP
UTP
ATP
ADP
0
0.1
1
10
100
1000
Agonist concentration (μM)

FIG.4